Inflammatory response to viral airway infections and secondary bacterial complications
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Chapter 1

General introduction
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Introduction

Influenza, the formal name for ‘flu’ is an uncommon term in both the English and Dutch language. Influenza originates from the Italian word for ‘influence’, which was derived from the medieval Latin word ‘influentia’ and gained its current meaning, ‘the power to produce effect’, in the sixteenth century. Later, the Italian word influenza was metaphorically used for outbreaks of diseases. In the nineteenth century, influenza or ‘flu’ became the common name for outbreaks of influenza virus [1]. The influence of this virus became clear in 1918, when an outbreak of influenza virus, referred to as the Spanish flu, killed over 20 million people [2]. Initially, it was thought that Pfeiffer’s bacillus (*Haemophilus influenzae*) was the causative organism of the Spanish flu. Shortly thereafter, it became clear that the disease was primarily induced by an ultra-filtrable pathogen [1]. This pathogen was isolated in the 1930’s and designated as influenza virus [3]. Nowadays, influenza virus is well known to increase the susceptibility for secondary bacterial pneumonia [4-6]. The excessive mortality rates during the Spanish flu were likely due to secondary bacterial complications [7]. Although bacterial complications have been observed for other respiratory viruses as well, these secondary infections are usually less severe [8, 9]. Viral infections have also been described to complicate chronic inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) [10, 11]. At present, respiratory viruses are appreciated for their impact (‘influence’) on airway inflammatory responses.

*Orthomyxoviridae*

Respiratory tract infection with influenza virus is well documented and often used as a model to study innate and adaptive immunity. Influenza virus is a negative-stranded RNA virus that belongs to the family of *Orthomyxoviridae* and can be divided into subtypes A, B and C [12]. Influenza virus is an enveloped virus with a diameter of 80-120 nm. The influenza A genome contains 8 segmented genes, which encode 10 viral proteins. The hemagglutinin (HA) and neuraminidase (NA) proteins expressed on the outer membrane are required to infect target cells. The matrix proteins (M1 and M2) provide rigidity to the virus. All other proteins play a critical role during viral replication [13]. The outer membrane protein play a critical role in host response after re-infection with influenza. Small variations in these proteins, i.e. antigenic drift, are due to single point-mutations. Single point-mutations easily occur in the influenza virus genome, since it lacks a proof-reading system. Large antigenic variations or
Antigenic shift are due to genomic recombination of two influenza virus strains [14]. At present 16 different HA proteins and nine NA proteins have been described [15, 16]. Antigenic shift is associated with severe outbreaks or pandemics of influenza virus, whereas antigenic drift is associated with milder outbreaks or epidemics of the infection. Pandemics occur every 12 to 20 years, whereas epidemics occur every winter season [4, 5, 14-16].

*Paramyxoviridae*

Parainfluenza virus infections are usually less severe than influenza virus infections [17]. Parainfluenza belongs to the family of *Paramyxoviridae*. Human parainfluenza virus (HPIV) can be divided into subtypes 1, 2, 3, 4A and 4B [12]. Sendai virus is the murine homologue of human parainfluenza type 1 [18]. Parainfluenza viruses are enveloped viruses with a diameter of 150-250 nm. The parainfluenza genome is composed of a single, negative RNA strand of approximately 15,000 bp and contains 6 genes. Some of these genes have multiple reading frames encoding for several proteins [18, 19]. Like influenza virus, HPIV and Sendai virus express a hemagglutinin-neuraminidase protein (HN) on their outer membrane. In contrast to influenza, paramyxoviruses require expression of a fusion (F) protein to penetrate target cells. The matrix protein provides rigidity and plays a critical role in the assembly of the virus [20]. Antigenic variation is much less common for human parainfluenza viruses and Sendai virus [21].

*Symptoms of influenza and parainfluenza airway infections*

Infections with influenza A usually cause symptoms such as fever, headache, sore throat, sneezing and nausea, accompanied by decreased activity and food intake. Influenza B closely resembles the illness induced by influenza A. Influenza C usually causes less severe ‘common cold-like’ symptoms [4]. The onset of symptoms is two to four days after infection and will last for 4 to 10 days. In rare cases influenza virus infection leads to pneumonia. Young children, elderly and immuno-compromised individuals are more susceptible for influenza virus infection and display a more severe outcome of the disease [22]. Parainfluenza virus normally causes upper respiratory tract symptoms, such as sore throat, running nose, sneezing and dry cough [19, 23]. Parainfluenza virus, especially HPIV-1 and HPIV-3, may cause pneumonia or bronchitis [24]. HPIV-1 and HPIV-2 have also been described to cause laryngotracheobronchitis (croup) [25]. The pathogenesis of HPIV-4 is still poorly understood. Recent studies indicate that the role of HPIV-4 in lower respiratory tract illness has been underestimated in the past due to poor diagnosis [26]. Both influenza and parainfluenza have
been associated with otitis media [27]. In rare cases, invasion by the influenza virus may lead to myocarditis/pericarditis or encephalitis [28].

Complications during viral airway infections
Although influenza virus may cause pneumonia, secondary bacterial pneumonia during and shortly after recovery from influenza virus infection is much more common [29]. The excess mortality rates during the pandemics of 1918-1919 and 1957-1958 can mainly be contributed to secondary bacterial complications [30]. Even nowadays, secondary bacterial pneumonia causes worldwide at least 40,000 deaths each year [31]. Bacteria such as *Staphylococcus aureus* and *Haemophilus influenzae* are known to cause post-influenza pneumonia, but *Streptococcus pneumoniae* is the most prominent pathogen causing secondary bacterial pneumonia in recent decades [4, 29, 32]. Primary infection with these pathogens are usually less severe than secondary infection [33]. Although secondary bacterial infections have been described for other respiratory viruses as well, including parainfluenza, the morbidity and mortality is much lower than observed for influenza virus [8, 9].

Viral infections may interfere with chronic airway inflammation, such as seen in asthma and COPD. Both influenza and parainfluenza have been implicated in asthma exacerbations, which are associated with increased airway hyperresponsiveness and enhanced inflammatory responses in the lower respiratory tract [34-37]. In contrast to asthma, COPD exacerbations are associated with bacterial infections, such as *Haemophilus influenzae, Streptococcus pneumoniae* or *Moraxella catarrhalis* [38-40]. However, viral infections, including influenza and parainfluenza, have been implicated as etiologic factor in acute exacerbations of COPD as well [36, 37]. Moreover, several studies indicate that viral airway infections predispose COPD patients to *H. influenzae*-induced exacerbations [41-43].

Innate and adaptive immune responses
Host defense against airway infections can be divided into innate immunity and adaptive immunity. Mechanical defense mechanisms, such as coughing, sneezing and mucociliary clearance are involved in the removal of inhaled pathogens [44, 45]. When pathogens escape these mechanical defense systems and invade the lower respiratory tract, an inflammatory response is induced leading to rapid recruitment of granulocytes and monocytes [45]. These inflammatory responses are initiated by recognition of these pathogens in the respiratory tract. Toll-like receptors (TLRs) are critically involved in the recognition of pathogen-associated
molecular patterns (PAMPs) [46]. At present, eleven TLRs have been identified, which recognize distinct PAMPs and detect microorganisms that express these PAMPs [46, 47]. For example, peptidoglycan interacts with TLR2 [48], lipopolysaccharide (LPS) with TLR4 [49], flagellin with TLR5 [50], whereas CpG-motifs in bacterial DNA activate TLR9 [51]. TLR1 and TLR6 function as co-receptor for TLR2 [52, 53]. Although the role of TLRs has been extensively studied for bacterial and fungal infections, TLR-mediated recognition of viruses is still poorly understood. TLR4 has been implicated in host defense against respiratory syncytial virus (RSV) [54, 55]. TLR4 has been shown to recognize the RSV fusion protein. Other important candidates to recognize negative-stranded RNA viruses are TLR3, TLR7 and TLR8, since these TLRs recognize either double-stranded or single-stranded RNA [56, 57].

After recognition by, and subsequent activation of these TLRs, an intracellular signaling cascade leads to activation of nuclear factor-κB and/or IRF-3 and the expression of pro-inflammatory mediators like cytokines and chemokines [58].

The array of cytokines and chemokines produced early after viral infection largely depends on the cell types that are infected by the virus. Both influenza virus and parainfluenza virus preferentially infect epithelial cells, although alveolar macrophages and recruited leukocytes may be infected as well [59]. Viral infection of airway epithelial cells leads to the release of, amongst others, interleukin (IL)-6, RANTES and IL-8. Pro-inflammatory mediators such as IL-1β and tumor necrosis factor (TNF)-α and chemokines such as IP-10 (interferon-γ inducible protein 10) and MIP-1α/β (macrophage inhibitory protein 1α/β) are primarily produced by alveolar macrophages [59, 60]. Chemokines play a critical role in the recruitment of granulocytes and monocytes to the lungs [61], while pro-inflammatory cytokines induce degranulation of these recruited cells [60, 62]. RSV and Sendai virus (mouse parainfluenza type 1) have been shown to activate neutrophil degranulation directly, whereas influenza virus inhibits granulocyte function [63, 64].

Antiviral mechanisms are triggered by the release of interferon (IFN)-α/β and IFN-γ by airway epithelial cells and/or alveolar macrophages. IFN-β binds to its receptor and triggers the expression of double-stranded RNA dependent protein kinase (PKR) [66], 2',5'-oligoadenylate synthetase (2-5A synthetase) [67] and myxovirus resistance proteins (Mx1 or MxA) [68]. PKR and 2-5A synthetase prevent the synthesis of viral proteins by means of translational blockade. Interestingly, influenza virus expresses a PKR-inhibiting protein, i.e. nonstructural
protein1 (NS1), thereby allowing it to escape from antiviral mechanism [69]. Mx1 (mouse) and MxA (human) are able to inhibit primary transcription and/or transport of viral mRNA. In addition, reduced availability of essential amino acids decreases total protein synthesis, which limits the production of viral proteins. For instance, expression of indoleamine-2,3-dioxygenase (IDO) during viral infection leads to decreased availability of tryptophan [70, 71] and results in reduced viral replication. Expression of IDO is mainly induced after release of IFN-γ by natural killer (NK) cells and/or T lymphocytes [72].

NK cells are activated early after infection. Due to their cytotoxic capacity, NK cells are able to target both extracellular and intracellular pathogens. NK cells are thought to play a central role in the innate immune response to viral infections. Although activation of NK cells is not dependent on antigen presentation, major histocompatibility complex (MHC) class I molecules play an important role in NK cell triggering [73]. MHC class I binds to inhibitory receptors on NK cells and prevent NK cell activation. In other words, NK cells target low MHC class I-expressing cells. It should be noted that NK cells also require an activation signal [74]. The role of activation receptors and the underlying mechanism of NK cell activation is poorly understood. Cytokines such as IL-12, IL-15 and IL-18 have been shown to promote NK cell activation as well [75].

Cytokines like IL-12 and IL-18 are primarily produced by (alveolar) macrophages [76, 77]. Although these cytokines have been shown to have a pro-inflammatory capacity, they are best known for their regulatory role in the adaptive immune response. Both IL-12 and IL-18 drive naive T helper lymphocytes towards a T helper type I (Th1) phenotype [78, 79]. Furthermore, IL-12 and IL-18 induce T cell proliferation and the release of typical Th1 cytokines like IFN-γ and IL-2 [79, 80]. Moreover, IL-12 and IL-18 synergistically enhance the release of IFN-γ by T lymphocytes [81]. IFN-γ plays an important role in the activation of cytotoxic T lymphocytes (CTLs), which are responsible for specific lysis of virus-infected cells [81-83]. Taken together, IL-12, IL-18 and IFN-γ are implicated in both innate and adaptive immune responses.

Cytokine release alone is not sufficient to induce T cell responses. Antigen presentation is required to provoke the formation of virus-specific T lymphocytes. T lymphocytes are activated if they recognize viral antigens loaded onto MHC class I or MHC class II molecules
Antigen-loaded MHC class I molecules bind to the T cell receptor (TCR) of CD8+ T cells (CTLs), whereas antigen-loaded MHC class II molecules bind the TCR of CD4+ T cells (T helper cells). DCs are the professional antigen presenting cells (APC), but other cells such as macrophages and monocytes may also function as APC [85, 86]. CD8+ T cells are particularly important for the killing of virus-infected cells, whereas CD4+ are generally regarded to have a helper function. However, recent studies indicate that CD4+ T cells can be cytotoxic as well leading to reduced viral loads in the lung [87].

After TCR triggering, a cascade of actions leads to the activation of T lymphocytes. An important aspect in the activation of T cells is the role of costimulatory molecules [88]. These costimulatory molecules do not only provide the signals required for expansion and cytotoxicity, they also play a role in driving the response towards Th1 or Th2 and thereby the activation of CTLs or B cells respectively [89]. Although CTL responses are generally believed to be most important for viral infections, B cells have been implicated in viral infections as well [90]. Viral hemagglutinin induces B cell proliferation. IL-6 release further enhances the proliferation of B cells and the differentiation into plasma cells, which leads to virus-specific immunoglobulin release [91]. During viral infection memory B and T cells are formed to prevent secondary infection with the same virus [92, 93].

Impaired host defense against secondary bacterial complications
A coordinated action of several innate and adaptive immune responses normally leads to appropriate eradication of the virus. Although the immune system is able to cope with viral respiratory infections, the host’s defense against secondary bacterial infections appears to be altered [4, 29-32]. Several mechanisms have been proposed to contribute to the enhanced susceptibility to bacterial infections [94]. Several bacteria release proteases that cleave the hemagglutinin protein on the outer membrane of viruses, thereby increasing the virulence of the virus [95]. However, increased virulence alone does not fully explain the enhanced susceptibility to bacterial infections, since individuals remain vulnerable for bacterial complications after recovery from influenza virus infection. A classical dogma is the enhanced colonization by bacteria of the airway submucosa due to disruption of the airway epithelial layer by the cytopathic effect of the virus [96]. Alternatively, increased colonization of bacteria may occur as a consequence of increased expression of attachment receptors. For instance, the platelet activating factor receptor (PAFR) has been suggested to play a critical
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role in colonization and invasion of *Streptococcus pneumoniae* [97]. However, clear evidence for a role of the PAFR or other attachment receptors is not yet available.

Several studies indicate that virus-infected individuals show an exaggerated inflammatory response upon stimulation by bacterial products such as staphylococcal enterotoxin B (SEB) and LPS [98-100]. Cytokines like IFN-γ, TNF-α and IL-6 are synergistically upregulated by SEB or LPS during influenza infections in mice [100]. These results indicate that the enhanced susceptibility to secondary bacterial infections, at least partially, depends on enhanced responses to secondary stimuli [101, 102]. The production of inflammatory mediators in virus-infected airway epithelial cells is based on both transcriptional and posttranscriptional regulation [103, 104]. Parainfluenza virus infection results in decreased degradation of mRNA, that are rapidly degraded, specifically those encoding response proteins. Secondary stimulation by either TNF-α or LPS leads to transcription of response genes, like IL-6 and IL-8. Increased transcription on one hand and reduced mRNA degradation on the other hand leads to increased expression levels of these messengers which is paralleled by enhanced IL-6 and IL-8 production. At present, the mechanism of virus-induced mRNA stabilization is not known. Several studies indicate that total protein synthesis plays a critical role in mRNA stabilization [103-106]. Total protein synthesis is reduced during viral infections in order to prevent biosynthesis of viral proteins [66-68, 103, 104]. Paradoxically, reduced total protein synthesis during viral infection in airway epithelial cells results in exaggerated IL-6 and IL-8 responses after secondary stimulation. Likewise, translational inhibition by cycloheximide leads to enhanced IL-6 and IL-8 production by airway epithelial cells upon stimulation by TNF-α or LPS [105]. It is thought that inhibition of total protein synthesis leads to reduced expression of mRNA degrading enzymes. One of the mechanisms by which viruses reduce total protein synthesis involves the tryptophan-catabolizing enzyme IDO [70, 71, 106]. The availability of tryptophan in the body is limited and therefore degradation of this amino acid results in reduced total protein synthesis. At present, it is not known whether IDO is directly induced by viruses or that IDO is expressed in an IFN-γ-dependent fashion. IFN-γ pretreatment of airway epithelial cells leads to the expression of IDO and results in enhanced production of inflammatory mediators after TNF-α or LPS stimulation [106]. Addition of tryptophan or methyltryptophan, an inhibitor of IDO, reversed this exaggerated inflammatory response in airway epithelial cells. Macrophages [107], DCs [108] and lymphocytes [109] have been shown to express IDO as well, but the
role of IDO in the potentiation of inflammatory responses has not been studied in these cells. Influenza-infected macrophages have been shown to increase the production of IL-1β, TNF-α and IL-6 in response to LPS, but the underlying mechanism depends on translation of preformed mRNA [101,110]. Alternative mechanisms may be responsible for the enhanced inflammatory response to secondary stimuli. These enhanced responses could also be the result of leukocyte recruitment to the pulmonary compartment after primary viral infection. However, the total number of recruited leukocytes declines rapidly after viral clearance, whereas individuals remain highly susceptible for several weeks after recovery from viral infection [94].

The enhanced susceptibility to secondary bacterial infections has been explained by reduced phagocytic capacity as well [102, 111]. Degranulation of neutrophils is impaired after influenza virus infection in vitro and in vivo, which results in enhanced bacterial outgrowth. The increased number of bacteria may explain the enhanced inflammatory response [112]. On the other hand, the release IL-10, an anti-inflammatory cytokine, may reduce the phagocytic capacity of these neutrophils too [113-115]. This latter mechanism illustrates the complexity of enhanced susceptibility to secondary bacterial infections during and shortly after recovery from influenza virus infections. Increased colonization, enhanced inflammation and reduced neutrophil function may all contribute to the pathology observed during secondary bacterial pneumonia. The explanations that are summarized here do not exclude each other.

Outline and scope of this thesis

A complex interaction between immune competent cells and the release of soluble mediators leads to clearance of respiratory viruses. At the same time, individuals become highly susceptible to secondary bacterial complications. The aim of this thesis is to obtain insight in the innate immune responses to primary viral infections and how these viral infections change the host response to secondary bacterial complications.

Mouse-models for pulmonary infection for influenza and parainfluenza (Sendai) virus were adopted to study innate immune responses to these viruses. Since RSV has been shown to induce cytokine release via TLR4, we hypothesized that other negative-stranded RNA viruses also activate this pathogen recognition receptor. The role of TLR4 during Sendai virus and influenza virus infection was studied in TLR4-mutant mice (chapter 2). The initial response to viral infection comprises recruitment of several inflammatory cells and the release of
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cytokines to activate these cells. Both IL-12 and IL-18 are pro-inflammatory cytokines involved in the induction of Th1 responses and thought to play a critical role in early host defense against viruses. The role of these cytokines against influenza virus infection was studied in IL-12 p35-gene deficient mice and IL-18 gene deficient mice respectively (chapter 3 and 4).

In the second part of this thesis, several important aspects of bacterial complications during and shortly after recovery from viral airway infections are discussed. In order to study these secondary bacterial infections, we extended the influenza mouse-model by administration of pneumococci shortly after recovery after influenza infection (day 14). This mouse-model for secondary bacterial infection is not biased by a concomitant viral infection, thus excluding direct interaction between the virus and bacteria. Pro- and anti-inflammatory cytokines contribute to the enhanced response to bacteria. The effect of IL-10, an anti-inflammatory cytokine, was studied by administration of a neutralizing antibody against IL-10 shortly before bacterial infection (chapter 5). The regulation of pro- and anti-inflammatory mediators by the tryptophan-catabolizing enzyme IDO was studied by implanting matrix-driven delivery pellets with the IDO-inhibitor methyltryptophan (chapter 6). The modified inflammatory response early after viral infection was studied by administration of LPS to Sendai virus-infected mice (chapter 7). LPS, a cell-wall component of gram-negative bacteria, was used to exclude interaction between influenza virus and bacteria. Combined infection of influenza virus and H. influenzae, a gram-negative bacterium, was performed to study the effect on outgrowth and/or clearance of both pathogens in vivo (chapter 8). Finally, attachment of pneumococci to the airway epithelium and the role of the PAF receptor in bacterial colonization during secondary pneumococcal pneumonia was studied in PAFR-gene deficient mice (chapter 9). Several aspects of secondary bacterial pneumonia, i.e. enhanced inflammation, reduced neutrophil function, increased colonization and direct interaction between virus and bacteria, are discussed in this thesis. Better understanding of the impact of viruses on host responses to bacterial infections may provide new targets to prevent or treat secondary bacterial complications.

References

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